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13. ABSTRACT ***(Maximum 200 Words)***

Citrus juices have many chemical components, we have shown to have anticancer properties, *in vitro* and *in vivo*. The principle agents investigated were the citrus limonoids: limonin, nomilin, and a limonoid glucoside mixture and the bioflavonoids: nobiletin, tangeretin, hesperetin and naringenin. Both sets of compounds were found to be potent inhibitors of cellular proliferation in estrogen receptor-negative (ER-) and -positive (ER+) cell lines. They were also found to act independently of the estrogen receptor. The bioflavonoids were shown to inhibit the growth of xenografted tumours in the nude mouse model. The effect of the limonoids in the treatment of an already established tumour in nude mice was investigated at their maximum tolerated dose. From histological analysis of mouse mammary tumours and cellular proliferation data it appeared that increased cell death was occurring. Therefore, to identify a mechanism of action for these compounds a FACS analysis was performed on cells treated with bioflavonoids and limonoids. Hesperetin, a bioflavonoid found in orange juice, induced apoptosis in MDA-MB 435 ER- cells. Naringenin from grapefruit juice had no effect. A limonoid glucoside mixture was also found to increase cell death, but the mode of action is still yet unclear.

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Introduction

In light of the continued high mortality due to breast cancer, the search continues for safe and effective antineoplastic agents. There is general agreement that plant-based diets, rich in whole grains, legumes, fruits and vegetables reduce the risk of various types of cancer, including breast cancer, and a variety of compounds produced by plants have been investigated for their anti-cancer activity (1-7). In particular, fruit products have been suggested as a source of a number of anti-cancer phytochemicals (8-10). Given the assumed safety of these compounds when ingested at levels found in a healthy diet, there is a potential to obtain a much higher therapeutic index using efficacious food extracts compared with traditional anticancer drugs. Identification of one or more of these phytochemicals having anti-tumour activity could yield an agent that would have important clinical usefulness.

Citrus fruits are a rich source of phytochemicals, with possible anti-cancer potential. These include the limonoids (Fig. 1), which are one of the two bitter principles found in citrus fruits, including oranges, grapefruits, lemons and limes. They are also present as glucose derivatives in mature fruit tissues and seeds, and are one of the major secondary metabolites present in citrus.

Limonoids have been shown to have anti-cancer activity. Nomilin reduced the incidence of and number of chemically-induced forestomach tumors in mice when given by gavage. Addition of nomilin and limonin to the diet inhibited lung tumor formation in mice and topical application of the limonoids was found to inhibit both the initiation and promotion phases of carcinogenesis in the skin of mice.

Previously our laboratory showed that citrus flavonoids (Fig. 2) and limonoids are effective inhibitors of estrogen receptor-negative (ER-) and receptor-positive (ER+) human breast cancer cells in culture. It was also shown that the flavonoids were able to delay tumour establishment and prevent metastasis in a nude mouse mammary-xenograft model where ER-, MDA- MB 435 cells were injected into the mammary fat pads of animals. Moreover, whole juices appeared to have a greater inhibitory effect compared to their constituent flavonoids, likely due to the combined actions of other components such as limonoids, vitamin C and hydroxy cinnamic acids.

BODY

Studies With Human Breast Cancer cells in Culture

Studies on the mechanism of Action: Although it is known that, the citrus limonoids and flavonoids are effective inhibitors of cellular proliferation in breast cancer cells not much is known about their mechanism of action. Histological analysis of tumours taken from mice treated with these compounds in their diets has indicated that an increased amount of apoptosis may be occurring compared to normal breast tissue. Therefore, to investigate this further, FACS analyses of MDA-MB435 cells incubated with limonoids or flavonoids at concentrations higher than their IC_{50}'s, were performed using the *Vybrant Apoptosis Assay Kit #2* from Molecular Probes. This assay involves the binding of Annexin-V to phosphatidyl serine molecules that translocate to the outer surface of the nuclear membrane during early apoptotic events. A second stain, Propidium Iodide (PI), is also used to determine cell membrane permeability. Viable cells undergoing apoptosis will be stained with Annexin-V only, dead cells that did not undergo apoptosis will be PI stained, whereas cells that underwent apoptosis and further degradation will be stained with both compounds. Using this method it was shown that all the citrus flavonoids and limonoids were capable of inducing apoptosis in MDA-MB435 cells to some extent (Fig.3) (Table 1). The most potent flavonoid observed was hesperetin with an increase of 14.99% in the apoptotic index, whereas the data for the limonoids is somewhat less clear (Table 1). Each limonoid was able to increase the apoptotic index within a range of 6 to 12% whereas the total percentage of doubly stained and Annexin-V stained cells is approximately 20%. DNA laddering assays must follow these experiments. Since DNA fragmentation is a hallmark of programmed cell death, this would confirm the induction of apoptosis by the flavonoids and limonoids and indicate which concentration apoptosis is first observed.

Following the physical identification of apoptotic cellular events changes in cell cycle regulation should be examined. Many flavonoids such as the soy isoflavones and silymarin have been shown to cause cells to exit the cell cycle and arrest the G1 phase. A flow cytometric technique for distinguishing cell cycle changes involves the incorporation of Bromodeoxyuridine (BrdU) into partially denatured DNA. BrdU/DNA distribution ratios demonstrate the relative amounts of BrdU incorporated into the DNA of cells whose DNA contents place them in the G_1, S, or G_2M phases of the cell cycle. By knowing where cells exit the cell cycle, insight can be gained into which pathways these compounds may affect. These studies are now in progress.

Experiments on cell proliferation: As previously stated, the effectiveness of the citrus limonoids and flavonoids has already been established in both the MCF-7 and MDA-MB-435 human breast cancer cell lines. Therefore, their ability to inhibit the proliferation of DU-145 and THP-1 human prostate and leukemia cells respectively was investigated. The compounds tested were the citrus flavonoids naringin, its glycoside naringenin, hesperetin, its glycoside hesperedin and the limonoids, limonin and nomilin as well a limonoid glucoside mixture. Cells were plated at a density of 2×10^4 cells/well in 96-well, flat-bottomed tissue culture plates in a total volume of 200 μL of medium and incubated at

37°C, with or without the test compounds. The plates were incubated for 48 hours at 37°C and [^{3}H] thymidine was then added to determine the number of dividing cells at each concentration. The cells were reincubated for 4 hours, after which the medium and excess radiolabel were removed. The cells were trypsinized and harvested onto a glass fiber filter paper, and the radioactivity was counted. The percentage of dividing cells was determined by comparing the number of disintigrations per minute of the treated cells (average of 3 wells/concentration) with that obtained for the control cells. The concentrations at which 50% growth inhibition occurred was determined (IC_{50}) for each compound (17). In DU-145 cells, the IC_{50}'s for the flavonoids were found to be approximately 29-31 μg/mL whereas in THP-1 cells the IC_{50}'s were in the range of 80-85 μg/mL. The limonoids have only been tested in DU-145 thus far and the IC_{50}'s for these compounds is in the range of 50-70 μg/mL (Fig.4).

Animal Studies

Animals. Female athymic nude mice (NCR-nu/nu), aged 3 weeks were housed in microisolator cages. The animals were maintained according to the "Guide for the Care and Use of Laboratory Animals," set by the Canadian Council on Animal Care (Ottawa) and adopted by The University of Western Ontario (London). They were randomly assigned to 1 of 3 experimental groups, with 12 mice in each group.

Diets. Each of the diets contained 5% corn oil (wt/wt). The diets were packaged in plastic bags and sealed. They were sterilized by irradiation and stored at -20°C until used.

Cell Line. The estrogen receptor-negative MDA-MB-435 human breast cancer cell line was maintained in minimum essential medium, alpha-modification, supplemented with 10% (vol/vol) fetal calf serum and grown to 80-90% confluence. The medium was changed 24 h before harvesting the cells by trypsinization. For injection, the cells were resuspended in phosphate-buffered saline (PBS) at a concentration of 20 x 10^6 cells/mL and kept on ice.

Treatment Groups. The citrus limonoids used in this study were limonin, and a limonoid glucoside mixture. They were incorporated into the diet at their maximum tolerated dose previously established (Table 2).

Experimental Procedure. The mice were anesthesized with metofane, and the tumour cells were injected into a right-sided mammary fat pad which had been exposed by a small incision. A 50 μL volume of inoculum containing 1 x 10^6 cells was injected and the incision was closed with skin clips. The mice were weighed and the inoculation site and auxiliary lymph nodes palpated at weekly intervals. When primary tumours became palpable, the maximum length and width of each were measured with callipers weekly until completion of the study and their surface areas were calculated. A tumour surface area of approximately 10.0 mm^2 was to be established before starting treatments to determine how effective the limonoids were at preventing further tumour growth and

metastasis. The mice were killed 8 weeks after beginning the treatments with limonoids. At necropsy, body weights and primary tumour weights were recorded and the number of macroscopic lung metastases was assessed as previously described. The auxiliary lymph nodes and lungs were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined by light microscopy to confirm the presence of metastases in these organs.

Body Weight and Growth of Mammary Fat Pad Tumours. The growth rate of the animals was similar in all of the groups. Initially a 90% success rate was achieved with regard to tumour establishment. However, after 4 weeks of treatment the tumours in the control group began to decrease in size along with the experimental groups (Figure 5). At the end of the experiment there were only 3 animals in the control group with tumours where only one was measurable and the number of surface lung metastases in the experimental groups was greater in comparison to the control group (Figure 6). Therefore, the decrease in tumour size for the animals given Limonin and the Glucoside Mixture cannot be attributed to the incorporation of these compounds in the test diets.

A possible explanation for the disappearance of control tumours in the study may be a result of natural killer (NK) and B cell activity in the nude mouse. In these mice only T_H cell populations have been compromised leaving them with partially intact immune systems. NK and B cells have been shown to have activity on cancer cell lines in athymic mice with specificity towards highly metastatic cell lines. Kim et al. (11) have shown that weekly administration of anti-NK cell and anti-B cell antibodies after cancer cell injection leads to almost 100% tumor establishment. Furthermore, ceasing antibody injections can lead to the arrest of tumour growth and tumour necrosis.

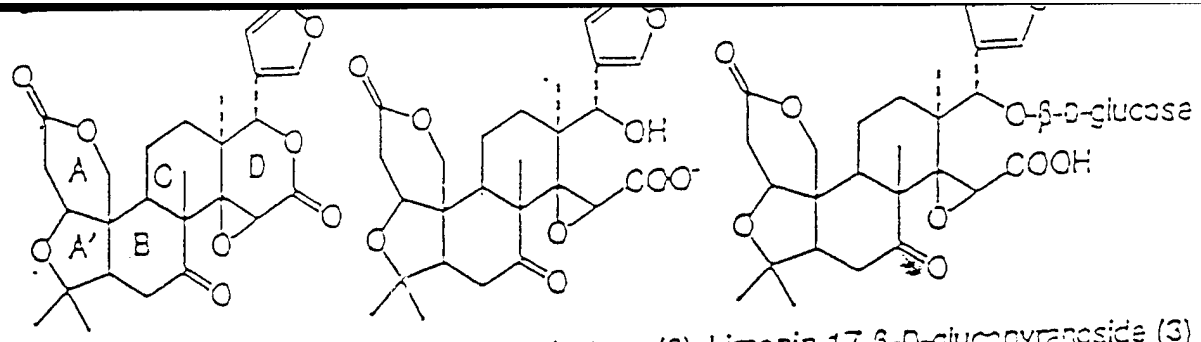
This experiment is now being repeated. The effect of citrus limonoids on ER+ tumors in nude mice is currently in progress.

Table 1: FACS Analysis Results for MDA-MB-435 Cells Treated with Citrus Flavonoids and Limonoids

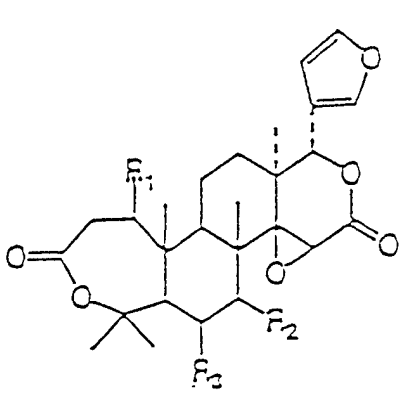
Compound	Conc(ug/mL)	% Apototic Cells LR	% Necrotic Cells UR
Controls			
No treatment		3.05	4.3
Actinomycin D		30.61	36.59
Flavonoids			
Hesperedin	70	4.48	8.72
Hesperetin	70	14.99	11.59
Naringin	35	9.91	7.39
Naringenin	35	6.25	9.72
Nobiletin	10	5.16	7.56
Limonoids			
Limonin	100	8.91	13.92
Nomilin	10	12.8	6.61
Glucoside Mix	10	6.39	12.53

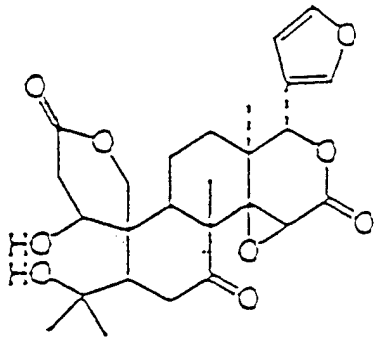
Table 2: Maximum Tolerated Doses for Citrus Limonoids in a 5% Semipurified Corn Oil Diet

Compound	***% Diet (w/w)***
Limonin	2
Glucoside Mix	4



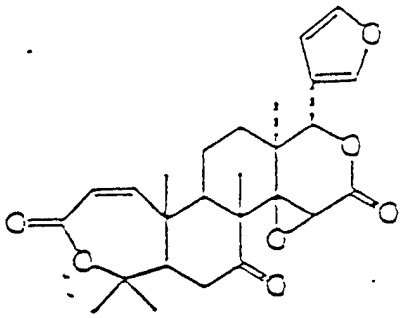
Limonin (1) Limonoate A-ring lactone (2) Limonin 17-β-D-glucopyranoside (3)

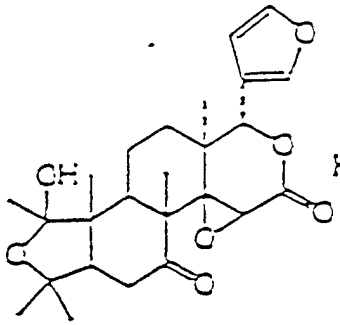


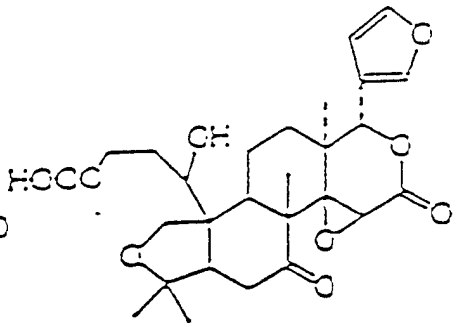


Ichangin (7)

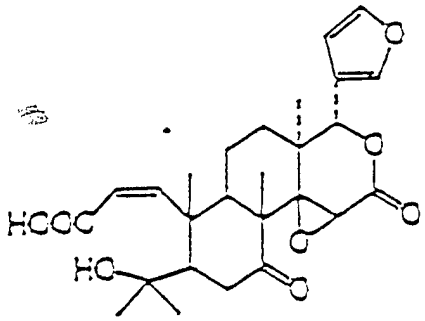
Nomilin (4) R_1=OAc, R_2=O, R_3=H
Deacetylnomilin (5) R_1=OH, R_2=O, R_3=H
6-keto-7β-Deacetylnomilol (6) R_1=CH, R_2=CH, R_3=O

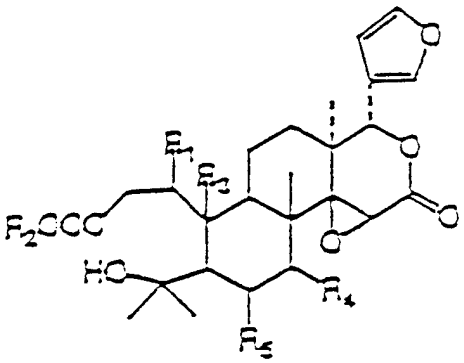






Obacunone (8) Ichangesin (9) Isolimonic acid (10)

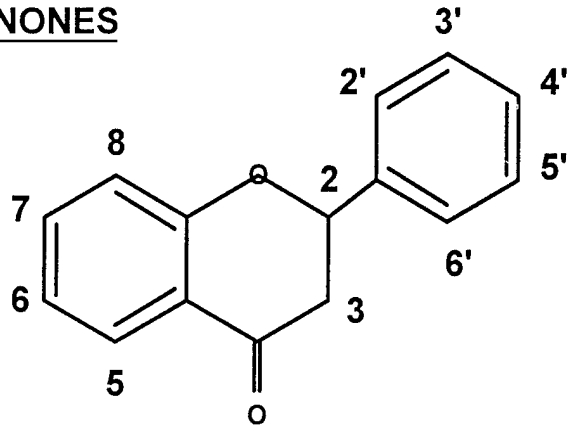




Obacunoic acid (11) Nomilinic acid (12) R_1=OAc, R_2=H, R_3=CH_3, R_4=O, R_5=H
Deacetylnomilinic acid (13) R_1=OH, R_2=H, R_3=CH_3, R_4=O, R_5=H
Calamin (14) R_1=OH, R_2=CH_3, R_3=CH_3, R_4=OH, R_5=O
19-Hydroxydeacetylnomilinic acid (15)
R_1=OH, R_2=H, R_3=CH_2OH, R_4=O, R_5=H

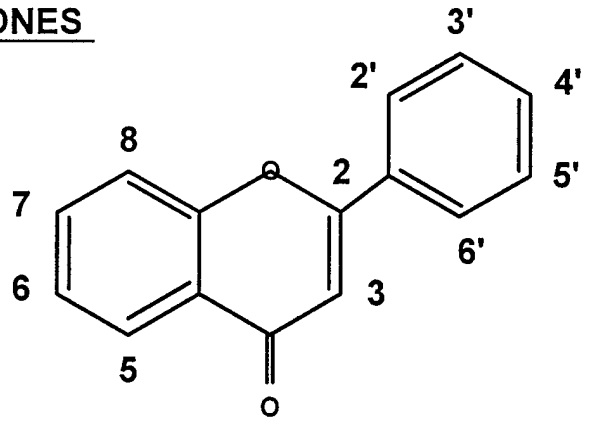
Figure 1: Structures of limonoids

FLAVANONES



	5	7	3'	4'
HESPERETIN	OH	OH	OH	O CH_3
NARINGENIN	OH	OH	—	OH

FLAVONES



	5	6	7	8	4'	5'
TANGERETIN	O CH_3	O CH_3	O CH_3	O CH_3	O CH_3	—
NOBILETIN	O CH_3	O CH_3	O CH_3	O CH_3	O CH_3	O CH_3

Figure 2: Structures of flavonoids

FACS Analysis

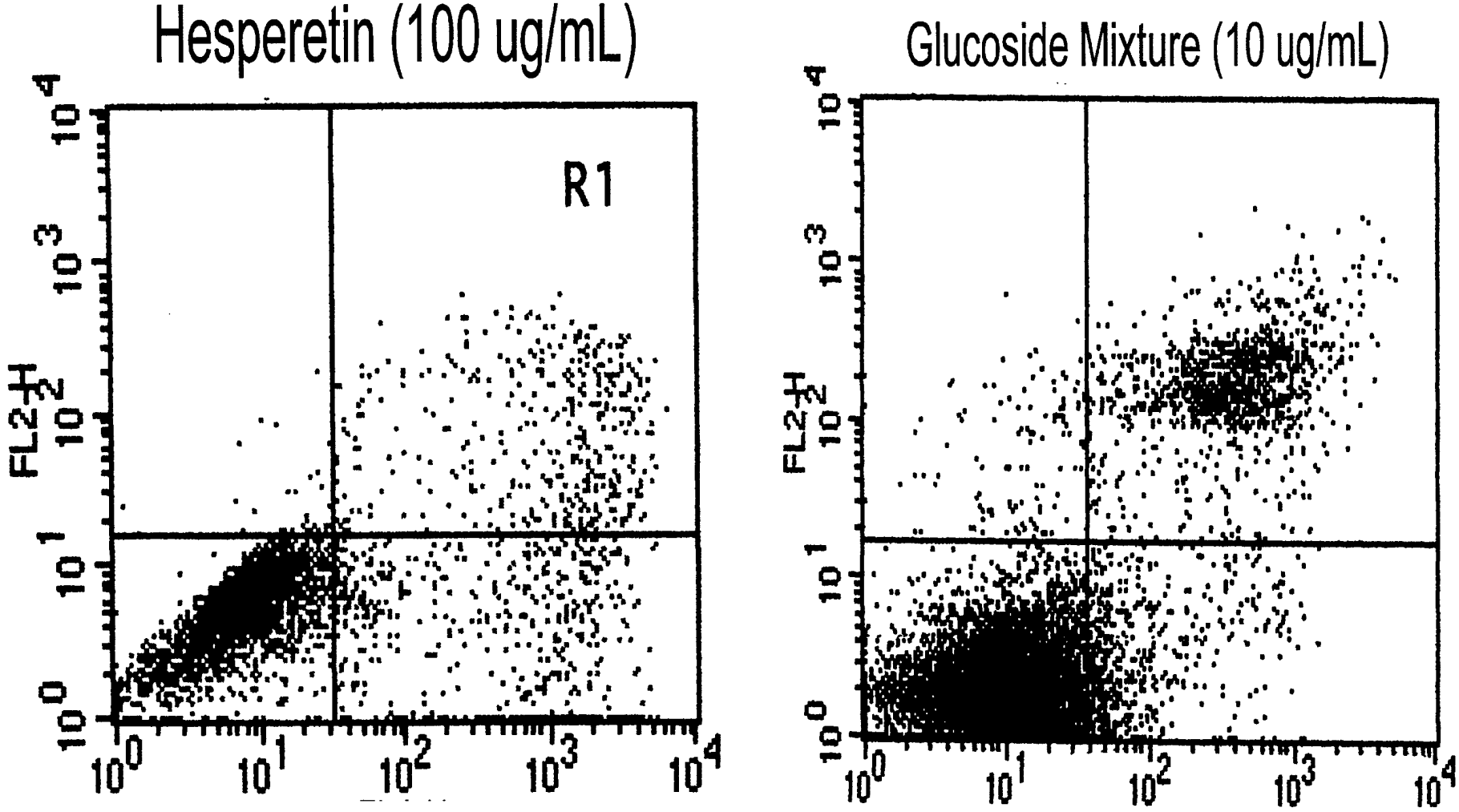


Figure 3: Representative Dot Plots for MDA-MB-435 cells treated with Hesperetin and The Limonoid Glucoside Mixture, respectively

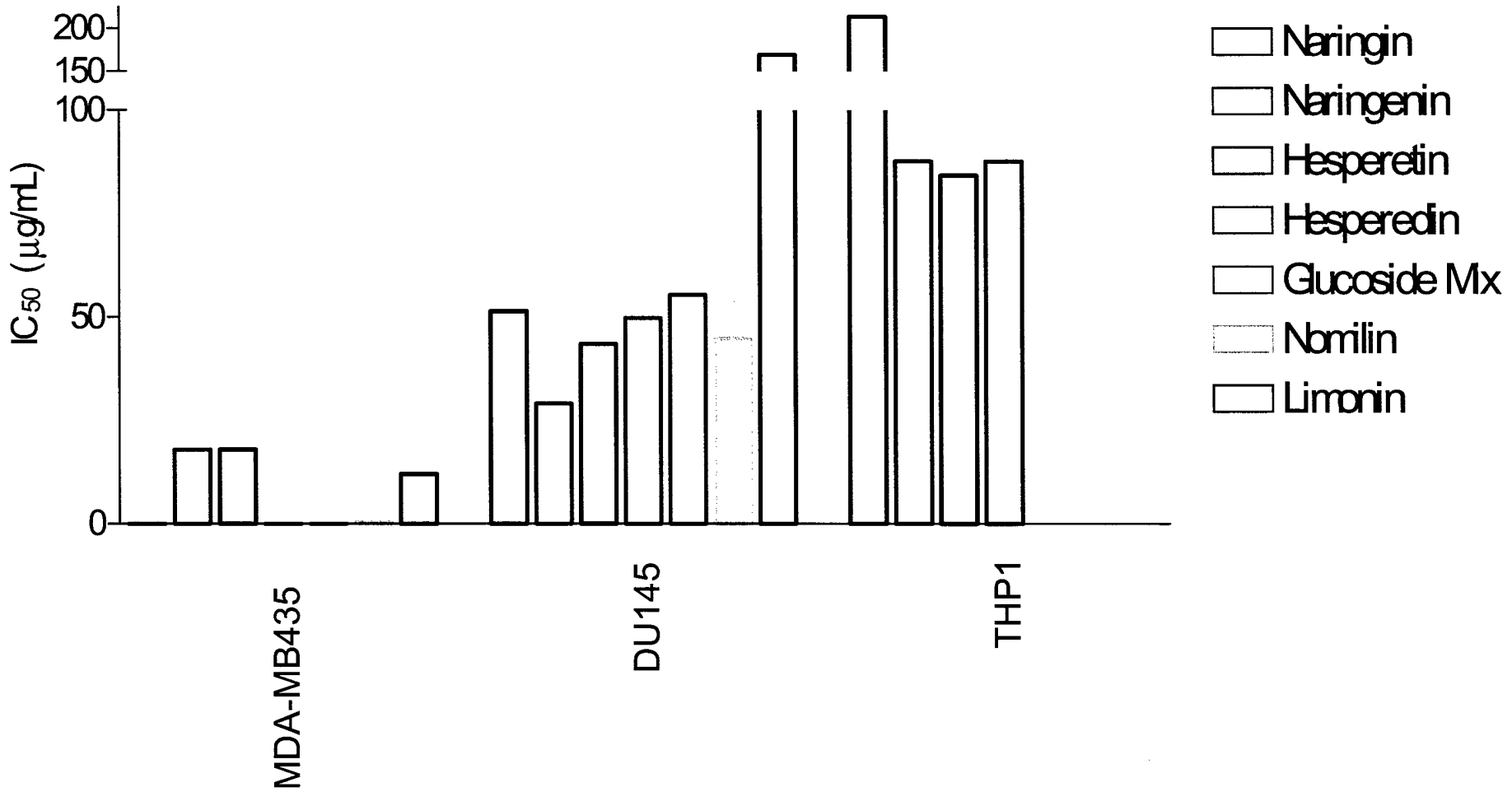


Figure 4: A comparison of the relative effectiveness of citrus juice components in 3 cell lines

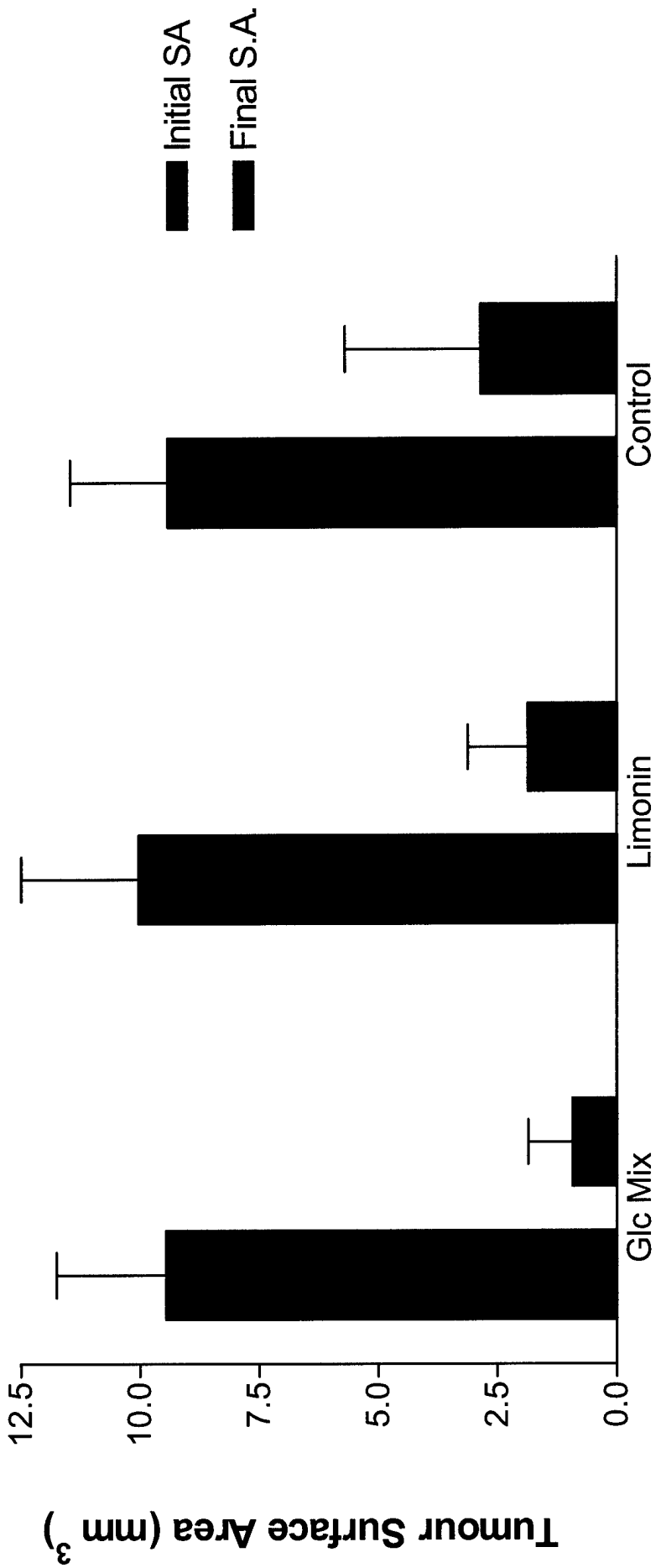


Figure 5:Initial Versus Final Tumor Surface Areas for Limonoid Treatment Study

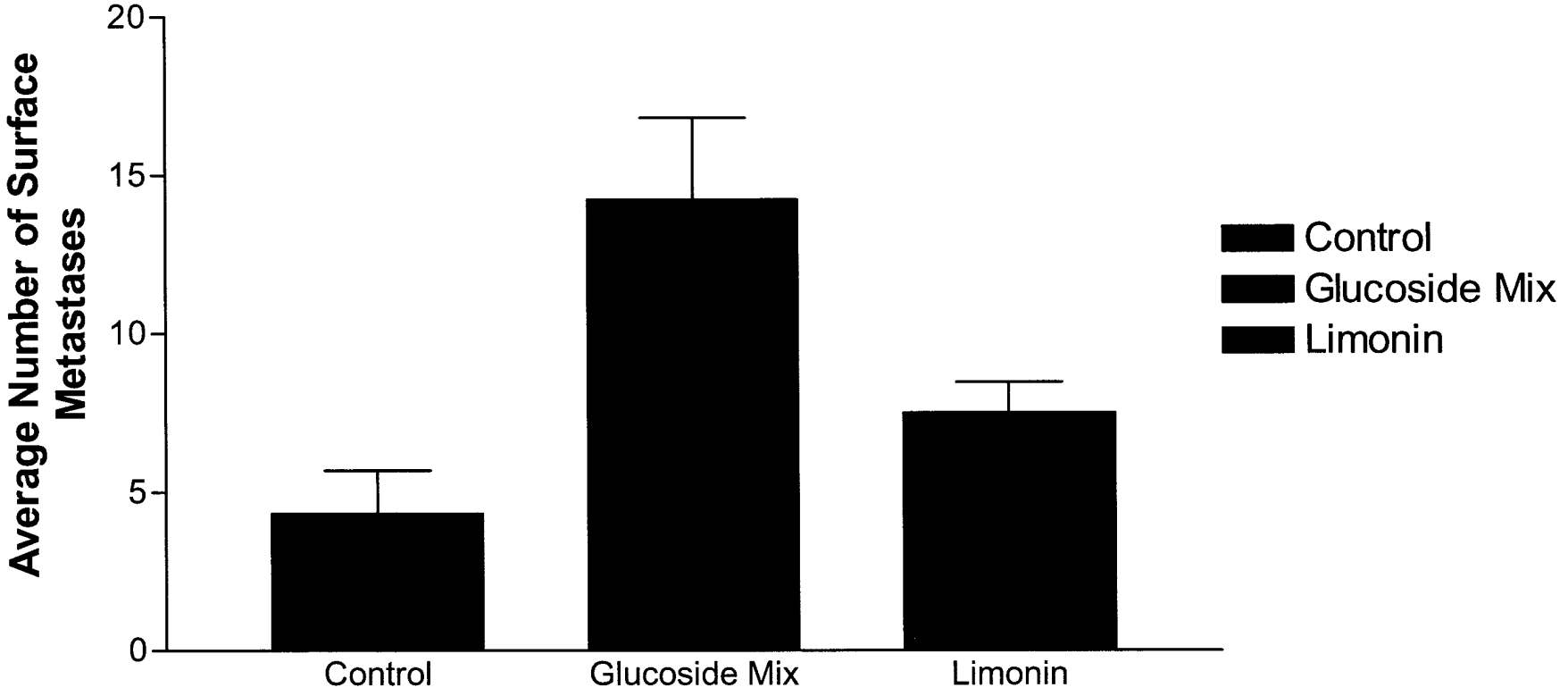


Figure 6: Average Number of Lung Surface Metastases Per Treatment Group

Key Research Accomplishments

- A number of naturally-occurring limonoids were screened for activity against other human cancer cells.
- The ability of the limonoids to induce apoptosis in MDA-MB-435 human breast cancer cells was investigated.
- One hundred grams of each of the limonoids were isolated.
- Diets containing maximum tolerated dose prepared and sterilized.
- Animal study investigating the effect of limonoids at their maximum tolerated dose on ER- tumors was carried out and repeated.
- Animal study investigating the effect of limonoids at their maximum tolerated dose on ER+ tumors is now in progress.
- Analysis of limonoids in tissues from animal studies is now in progress.
- Presented data at Cancer Care Ontario "Hormone Dependent Tumours: Biology, Prevention and Therapy" conference, Lake Couchiching, Ontario, November, 1999.
- Presented data at Experimental Biology, San Diego, CA, April, 2000.
- Presented data at Breast Cancer Research Program Era of Hope, Department of Defense, US Army Medical Research and Materiel Command, Atlanta, Georgia, June, 2000.
- Invited speaker at the Breast Cancer Translational Seminar Series, London Regional Cancer Centre, London, Ontario, June, 2000.

Reportable Outcomes

1. Guthrie, N., Hasegawa, S., Manners, G., Lippman, M.E., Clarke, R. & Vandenberg, T.A. Effect of citrus limonoids on human breast cancer cell growth in culture and in nude mice. Proc. Department of Defense, US Army Medical Research and Materiel Command, Breast Cancer Research Program, Era of Hope Atlanta, Georgia, June 8-12, 2000 (Abstract).

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5. Freeman D.J. & Guthrie, N. Anti-Cancer Properties Of Citrus flavonoids and limonoids. Grant applied for and received from the Academic Development Fund, The University of Western Ontario, ($7,400.00).

Conclusions

We have previously reported that citrus flavonoids inhibit proliferation and growth of estrogen receptor-negative (ER-) and -positive (ER+) human breast cancer cells in culture and that orange juice inhibits chemically-induced mammary carcinogenesis in rats. In addition, we investigated the effect of citrus limonoids on human breast cancer cells and found them to be potent inhibitors of poliferation of ER- & ER+ cells.

Our results on the mechanism of action have shown that citrus limonoids and flavonoids increased the percentage of apoptotic cells after 24 hours in MDA-MB-435 cell preparations. They are able to inhibit both DU-145 and THP1 cells to a lesser extent than MDA-MB-435 cells. In the studies on nude mice, we found that although ER- tumours regressed in both animal study treatment groups no definitive answer can be gained from the current data. This experiment is currently being repeated.

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